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(21) International Application Number: PCT/DK91/00308 (22) International Filing Date: 10 October 1991 (10.10.91) (30) Priority data: 2451/90 10 October 1990 (10.10.90) DK 773/91 25 April 1991 (25.04.91) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : BUCH-RASMUSSEN, Thomas [DK/DK]; Dalvej 28, DK-2820 Gentofte (DK). OLSEN, Bjarke, Rosenblad [DK/DK]; Markvej 52, DK-2660 Brøndby Strand (DK). KULYS, Juozas [SU/SU]; Institute of Biochemistry, Lithuanian Academy of Sciences, Vilnius-MTP (SU). BECHGAARD, Klaus [DK/DK]; Åbovænge 4, DK-2610 Rødovre (DK). CHRISTENSEN, Jørn, B. [DK/DK]; Meinungsgade 12, 2.tv., DK-2200 Copenhagen N (DK). WANG, Joseph [US/US]; 1736 Salinas Drive, Las Cruces, NM 88001 (US). OZSOZ, Mehmet (Sengun) [TR/TR]; 1725 Sokak No: 44/3, Karsiyaka, Izmir (TR). COLIN, Fernan [BE/BE]; GARCIA, Orféla [BE/BE]; Université de Médecine et de Pharmacie, Laboratoire d'Electrophysiologie, Boulevard de Waterloo 115, B-Bruxelles (BE).		(74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU ⁺ , TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i>
(54) Title: USE OF BENZENE DERIVATIVES AS CHARGE TRANSFER MEDIATORS (57) Abstract Benzene derivatives having optionally substituted amino or morpholino in the 1 and 4 position, optionally alkyl in the 2, 3 and 6 position and optionally alkyl or nitro in the 5 position or wherein the 1 and 6 substituents together form a heterocyclic ring, can be used as a charge transfer mediator in a biochemical process involving electron transfer between a redox system and an electrode comprising a paste of electrically conductive particles and a pasting material.		

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USE OF BENZENE DERIVATIVES AS CHARGE TRANSFER MEDIATORS

FIELD OF THE INVENTION

The present invention relates to a biochemical method for measuring the concentration of at least one constituent of a fluid sample, especially glucose in a glucose
5 containing aqueous medium, for example a body fluid, in particular a whole blood sample and to charge transfer mediators for use in such a method.

DESCRIPTION OF PRIOR ART

Electrode devices and methods for detecting the
10 amount of selected compounds in a liquid mixture are known in the art. Thus, in European patent application having publication No. 78,636B and 125,137A a sensor electrode for in vivo measurement of the amount of a component in a liquid mixture is described. Therein, a charge transfer
15 mediator, such as ferrocene, is used in an enzyme catalyzed reaction to transfer electrons arising from the reaction between the enzyme and a component in the liquid. The charge transfer mediator (hereinafter for the sake of brevity designated mediator) is adhered to the surface of the
20 electrode in the form of a thin film layer or strip, for example by deposition from a solution. This known device suffers from the severe drawback that the mediator during the measurement rather rapidly will be depleted from the strip, causing a deterioration in measurement reproducibility.
25 A similar device is described in European patent application having publication No. 127,958A. The electrode devices are preferably intended for in vivo measurement of glucose in blood.

International patent application having publica-
30 tion No. WO 89/10395 describes an enzyme electrode comprising a matrix made from a conductive powder, an enzyme and a mediator. The device suffers from the drawback that the concentration of the mediator is rather low. It is therefore necessary to operate the electrode at a rather
35 high applied potential, leading to interference from other oxidizable substances in the sample.

International Patent Application No. PCT/DK90/00067 describes a method of measuring at least one constituent of a fluid sample by means of an electrode device including at least one measuring electrode having an exposed surface part at a free end portion of the electrode device and extending longitudinally through said end portion, comprising removing an outer end section of the free end portion so as to provide a new exposed surface part of the measuring electrode, and subsequently exposing said exposed surface part of the measuring electrode to a sample of said fluid, so as to generate a measuring signal representative of said constituent of the sample.

The method according to International Patent Application No. PCT/DK90/00067 can be used for measuring the concentration of a constituent of gaseous and liquid fluid samples of any type. The method may in particular be used for measurements on fluid samples within the medical and veterinary field. Thus, the fluid sample may, for example, be a sample of animal or human body fluids, such as whole blood. The known method is especially useful in connection with the measurement of the content of glucose in blood and is based on an electrochemical principle using a mediator present in a non-polar medium in a sensor electrode which operates at a constant potential which is sufficiently low to avoid interference resulting from oxidation of other oxidizable substances, such as acetylsalicylic acid (aspirin), paracetamol (acetaminophen), ascorbic acid, and sulfonylureas (tolbutamide, glibenclamide), present in the sample. The method is especially advantageous in that a specific measure of the concentration of glucose is obtained without any significant uncertainty arising from a co-determination of interfering substances which, in general, can be present in the blood. The method is based on monitoring the flow of current at an electrically conductive sensor electrode when glucose oxidase catalyzes a redox reaction of glucose in the presence of a mediator.

International Patent Application No. PCT/DK90/00067 also describes an electrode device comprising an electrode body member (electrode device body) having a free end portion, and at least one measuring or
5 sensor electrode extending axially through said end portion and having a substantially uniform cross-sectional area within said end portion. Because the cross-sectional area of each of the sensor or measuring electrodes positioned in the free end portion of the electrode body member is sub-
10 stantially uniform, the exposed active surface area of each electrode remains substantially unchanged when an outer end section or slice is removed from the free end portion. The electrode body member in which the measuring electrode is embedded may in itself define an outer electrode provided
15 that this outer electrode is sufficiently electrically insulated from the other inner electrode or electrodes.

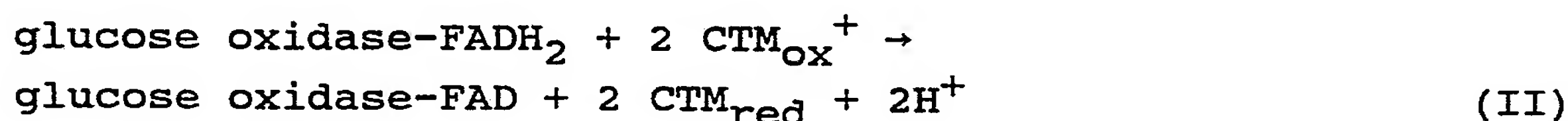
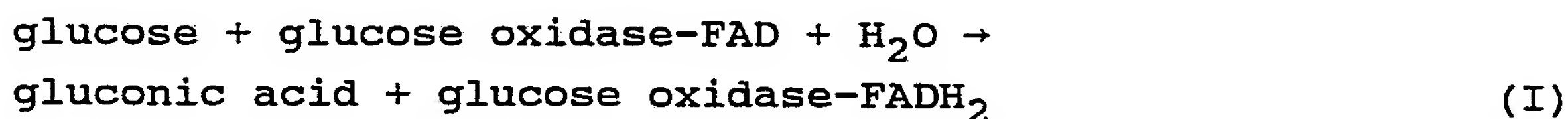
The electrode device comprises an electrode device body, a sensor electrode, a reference electrode and a suitable counter electrode. The electrodes are positioned in
20 separate channels in the electrode device body having the shape of a cylindrical rod, and the electrode channels are longitudinally disposed in the electrode device body.

The body of the electrode device is made of a sliceable polymeric material and the sensor electrode
25 ingredients are uniformly dispersed in a substantially water-insoluble carrier medium and uniformly distributed throughout the channels in the form of a sliceable, high-viscosity paste such as paraffine. This construction permits the reuse of the electrode device for a number of
30 determinations, a fresh sensing surface being provided before each determination by cutting away a cross-sectional slice. The measurement is performed by bringing a surface of the electrode device, comprising cross sections of all the electrodes present, into contact with a sample of the
35 drop of whole blood, which is taken from a patient, preferably by the patient himself, before the measurement is to be made. Before every measurement, the sensing sur-

face of the electrode device is renewed by cutting off a thin slice of the electrode device in order to ensure the removal of any contaminants present on the sensing surface and to provide a fresh sensing surface of the electrode device. In addition, the removal of the sensor electrode sensing surface which has been exposed to the aqueous medium in question during the preceding measurement serves to ensure that the required amount of glucose oxidase and mediator is present at the surface of the electrode(s).

The basis of the method for measuring the concentration of glucose in a sample of a glucose-containing aqueous medium, which can be performed by means of an apparatus comprising the above electrode device is the reaction between glucose and the enzyme glucose oxidase (GOD). Electrons produced by this oxidation reaction are transferred to a mediator and finally captured by a conductive material in the sensor electrode, for example graphite particles. The latter enzyme-catalyzed oxidation of glucose leads to gluconic acid and the "reduced form" of glucose oxidase, i.e. glucose oxidase wherein at least one functional group has been reduced. The reduced enzyme reacts with the oxidized form of the mediator, which is formed by donation of an electron from the "reduced" form of the mediator to the electrically conductive sensor electrode, and the glucose oxidase is thereby regenerated together with the reduced form of the mediator. The electrical current produced in the process is proportional to the concentration of glucose present in the sample.

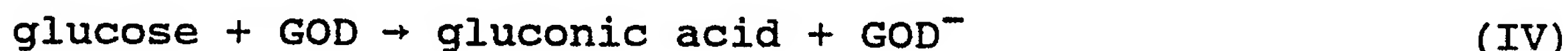
The reactions can schematically be described by the following equations:





where FAD designates the oxidized form of the flavin-adenine dinucleotide part of glucose oxidase, FADH_2 designates the reduced form of the flavin-adenine dinucleotide part of glucose oxidase, $\text{CTM}_{\text{ox}}^{+}$ designates the mediator in oxidized form and CTM_{red} is the mediator in reduced form.

A simplified way of expressing the reactions is:



Electrodes based on the above general principle are known, but they suffer from problems caused by the depletion of the mediator, due to relatively high water-solubility of the oxidized form thereof.

15 A significant extent of depletion of the mediator can result in several drawbacks, for example

- (i) only one measurement can be performed in a given sample owing to rapid loss of the mediator to the medium of the sample;
- 20 (ii) the reproducibility of the measurement is poor as the extent of the depletion of the mediator is rather unpredictable and depends on the time elapsing between exposure of the sample to the electrodes in question and the performance of the actual measurement;
- 25 (iii) the sensitivity at low glucose concentrations is very poor; and
- (iv) the applied potential between the sensor electrode and the reference electrode has to be rather high, leading to interference from other oxidizable substances present in the aqueous sample.
- 30

A prerequisite for a successful and reproducible measurement of the specific glucose concentration in a body fluid, in particular in whole blood, based on the above-mentioned principle using a suitable apparatus comprising the sensor
5 electrode, a suitable reference electrode and a suitable counter electrode is thus that a sufficiently well-defined amount of the mediator is present throughout the measurements.

The amount of the mediator present in the sensor
10 electrode is a very important feature of the present invention. The amount should throughout the lifetime of the electrode device be sufficiently high to ensure that the concentration of the oxidized form of the mediator in the aqueous sample is not limiting the enzyme reaction during
15 the measurement.

DESCRIPTION OF THIS INVENTION

The present invention is related to a method based on the above mentioned electrochemical principle for measuring the concentration of certain compounds such as
20 glucose, theophyllin, alcohol (ethanol), lactate or cholesterol, in a sample of an aqueous medium, for example a sample of body fluid, such as a sample of whole blood, blood plasma, serum, urine or saliva, in which certain groups of mediators are used. The mediators are characterized by having a low solubility in their reduced form in
25 water, for example lower than 10 mM.

This ensures that the mediator is present and available in an amount sufficient to ensure that the concentration of its oxidized form is maintained substantially
30 constant during the measurement.

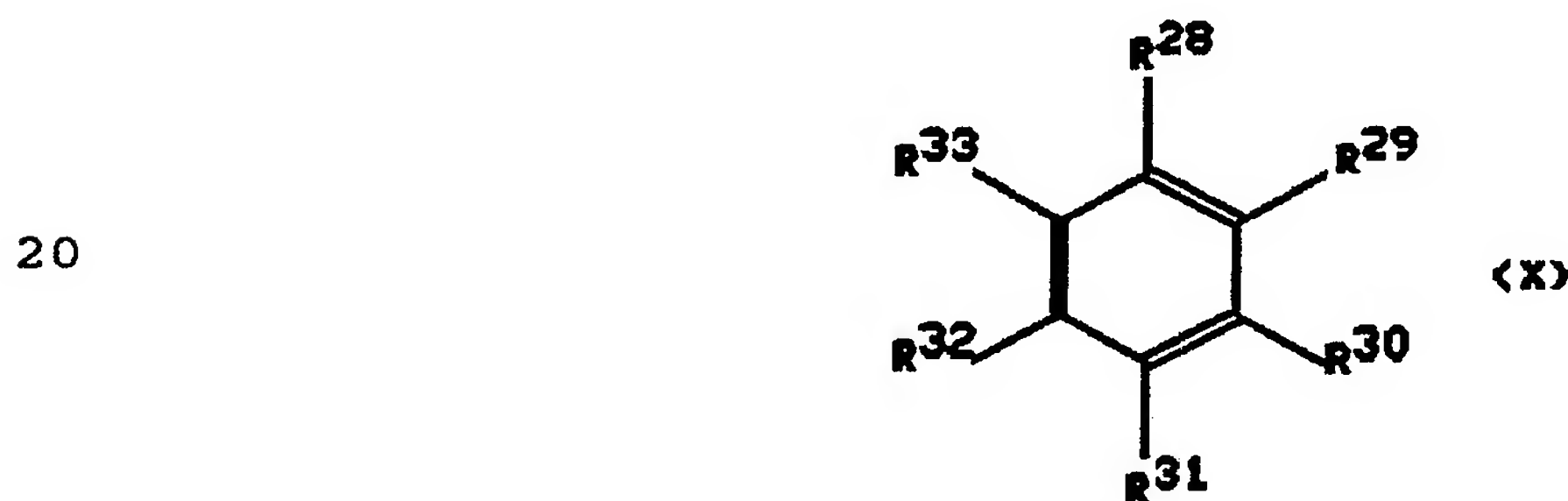
Hence, this invention relates to a biochemical process involving electron transfer between a redox system and an electrode comprising a paste of electrically conductive particles and a pasting material and a charge transfer
35 mediator, wherein the mediator has a low water solubility.

In one aspect of this invention, the mediator has a high solubility in the pasting material. In the pasting material, the mediator may wholly or partially be present in crystalline form. The pasting material may be a non-polar oil such as paraffin oil.

The mediator should have the following characteristics:

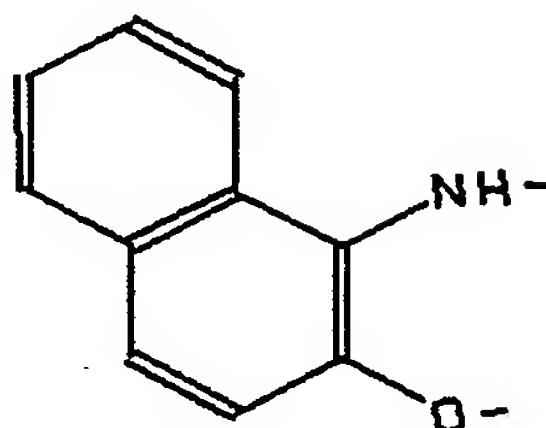
- 1) A low solubility in water in the reduced form.
 - 2) An oxidation potential between -300 and +400 mV versus an Ag/AgCl reference electrode.
 - 10 3) The reaction rate of the mediator in the enzyme reaction and the electrode reaction must not be the limiting step in the total reaction rate.
 - 4) It must be stable.
 - 5) It must not participate in other reactions.
- 15 The present invention is also related to mediators for use in the method described above. Additionally, this invention relates to the use of compounds as mediators.

It has now, surprisingly, been found that compounds of the general formula X

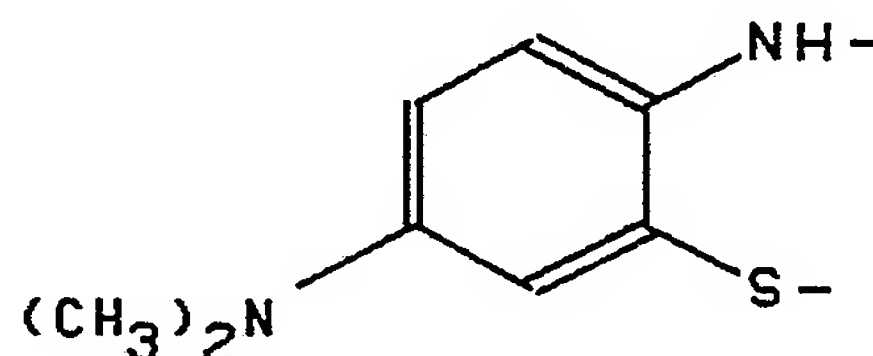


wherein R²⁸ and R³¹ are the same or different and each represents amino, alkylamino, dialkylamino, morpholinyl or piperidyl, and R²⁹, R³⁰ and R³³ are the same or different and each represents hydrogen or alkyl, preferably hydrogen, 25 R³² represents hydrogen, alkyl or nitro, preferably hydro-

gen or nitro, or R^{28} together with R^{33} either represents a moiety of the formula Xa



wherein the amino group is in the R^{28} end, or R^{28} together with R^{33} represents a moiety of the formula Xb



5 wherein the amino group is in the R^{28} end, fullfil the above, strict requirements for being a good mediator.

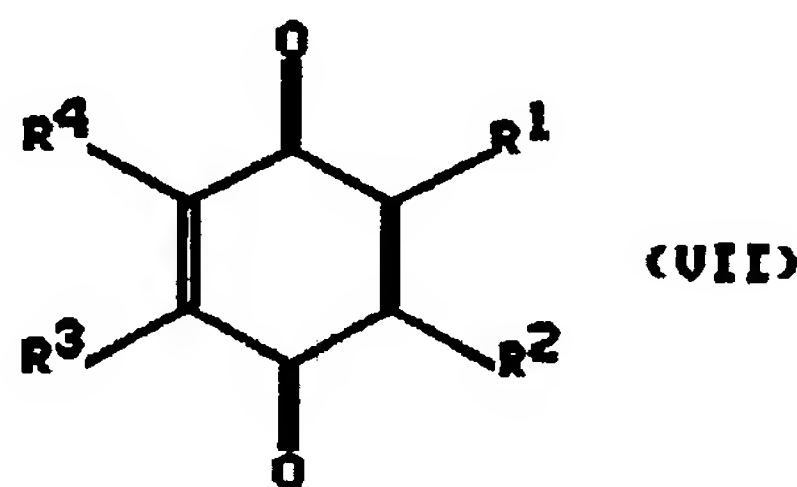
Especially preferred compounds of formula X are compounds wherein R^{28} and R^{31} are the same or different and each represents amino, alkylamino, dialkylamino, morpholi-
 10 nyl or piperidyl, and R^{29} , R^{30} , R^{32} and R^{33} are the same or different and each represents hydrogen or alkyl, preferably hydrogen.

Examples of specific, preferred compounds of formula X are 1,4-diamino-2,3,5,6-tetramethylbenzene, 1,4-bis-
 15 (dimethylamino)benzene, 1-dimethylamino-4-morpholinobenzene, 1,4-dimorpholinobenzene, 1-morpholino-4-piperidinobenzene, 2,5-dimethyl-1,4-bis(dimethylamino)benzene, 1,4-dipiperidinobenzene, N-(4-morpholinophenyl)hexahydroazepine, 4-nitro-3,7-bis(dimethylamino)phenothiazine (-
 20 methylene green) and Meldola's Blue (= 7-dimethylamino-1,2-benzophenoxazinium salts, for example chloride or sulphate).

Herein, the term alkyl is preferably lower alkyl which may be straight or branched and which preferably contains not more than 10 carbon atoms, more preferred not more than 6 carbon atoms. Analogous comments relates to groups containing an alkyl group such as alkylamino and dialkylamino.

Compounds of formula X are either known compounds or compounds which can be prepared in analogy with the preparation of known compounds.

In addition to the above compounds of formula X, also compounds of the general formula VII:



wherein R^1 , R^2 , R^3 and R^4 are the same or different and each represents alkyl or alkylene with up to 10 carbon atoms, acyl with up to 10 carbon atoms, aryl optionally substituted with alkyl, halogen or nitro, alkoxy with up to 10 carbon atoms, hydroxy, halogen, amide, alkyl amide with up to 10 carbon atoms, aryl amide, mercapto, alkyl thio with up to 10 carbon atoms, aryl thio or hydrogen, with the proviso that at least one of R^1 , R^2 , R^3 and R^4 is different from hydrogen, or R^1 and R^2 together represents a moiety of the general formula VIIa

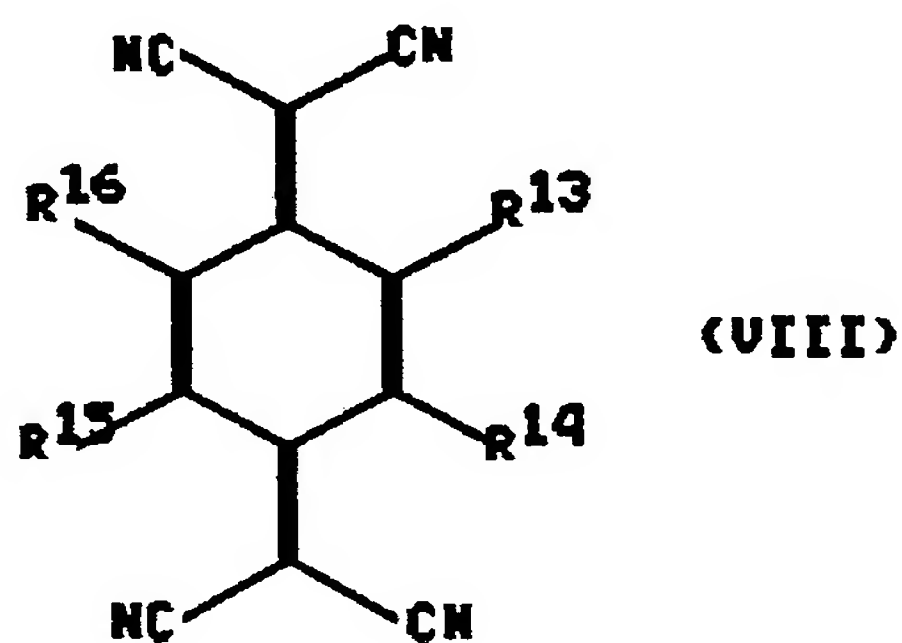


wherein R^5 , R^6 , R^7 and R^8 are the same or different and each represents hydrogen, alkyl or alkylene with up to 10

carbon atoms; acyl with up to 10 carbon atoms; aryl optionally substituted with alkyl, halogen or nitro; alkoxy with up to 10 carbon atoms; hydroxy, halogen, amide, alkyl amide with up to 10 carbon atoms, aryl amide, mercapto, alkylthio
 5 with up to 10 carbon atoms or arylthio, or R^3 and R^4 together represents a moiety of the general formula VIIb



where R^9 , R^{10} , R^{11} and R^{12} are the same or different and each represents hydrogen, alkyl or alkylene with up to 10
 10 carbon atoms; acyl with up to 10 carbon atoms; aryl optionally substituted with alkyl, halogen or nitro; alkoxy with up to 10 carbon atoms; hydroxy, halogen, amide, alkyl amide with up to 10 carbon atoms, aryl amide, mercapto, alkylthio with up to 10 carbon atoms or arylthio, with the proviso
 15 that formula VII does not cover 2-methyl-p-benzoquinone, tetrachloro-p-benzoquinone, 2,5-dimethyl-p-benzoquinone, 2-isopropyl-5-methyl-p-benzoquinone, tetramethyl-p-benzoquinone, tetrabromo-p-benzoquinone, 2,5-dichloro-p-benzoquinone, 2,6-dichloro-p-benzoquinone, α -naphthoquinone, β -
 20 naphthoquinone and β -naphthoquinone-4-sulfonate, compounds of the general formula VIII

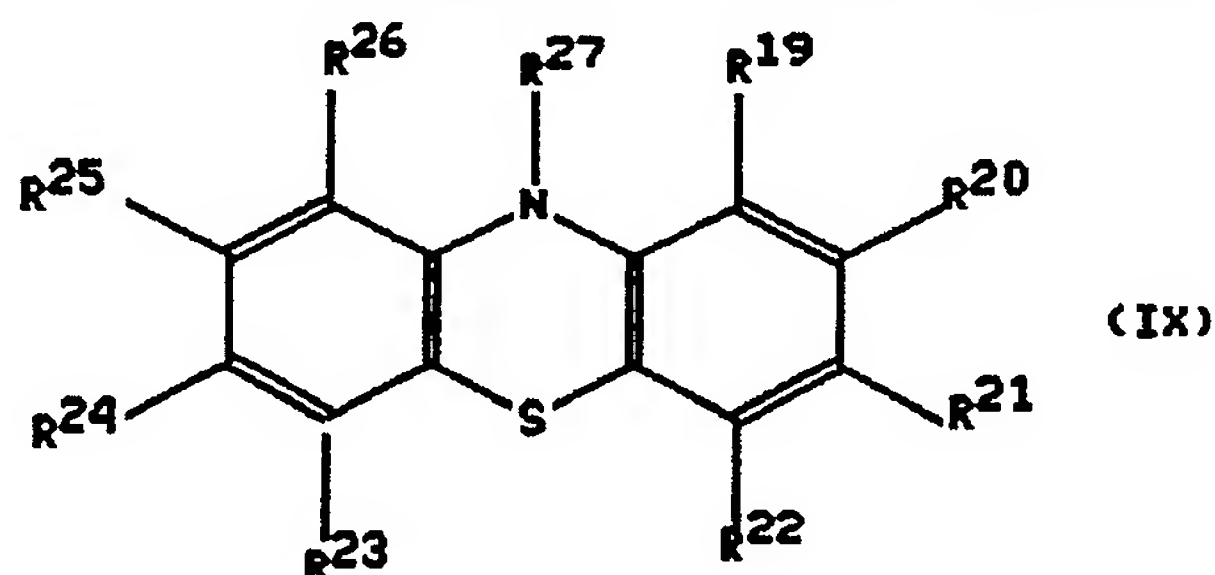


wherein R^{13} , R^{14} , R^{15} and R^{16} are the same or different and each represents hydrogen, alkyl or alkylene with up to 10

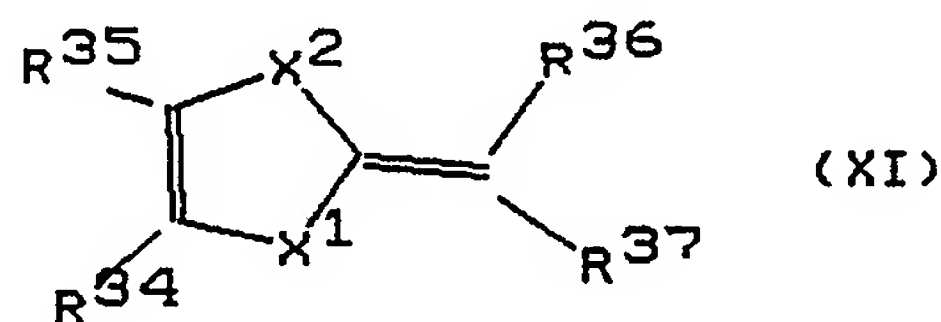
carbon atoms, aryl, alkoxy with up to 10 carbon atoms or acyl with up to 10 carbon atoms, with the proviso that at least one of the symbols R^{13} , R^{14} , R^{15} and R^{16} is different from hydrogen, or wherein R^{13} and R^{14} together forms a moiety of the general formula VIIIIa



wherein R^{17} and R^{18} are the same or different and each represents hydrogen, alkyl or alkylene with up to 10 carbon atoms, aryl, alkoxy with up to 10 carbon atoms or acyl with up to 10 carbon atoms, compounds of the general formula IX

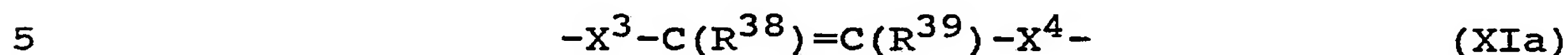


wherein R^{19} , R^{20} , R^{21} , R^{22} , R^{23} , R^{24} , R^{25} and R^{26} are the same or different and each represents hydrogen, nitro, halogene (preferably chloro), amino, alkylamino, dialkylamino, or morpholinyl, and R^{27} represents hydrogen, alkyl or dialkylaminoalkyl, and compounds of the general formula XI



wherein X^1 and X^2 are the same or different and each represents sulphur or selenene, and R^{34} and R^{35} are the same or

different and each represents hydrogen, alkyl, alkoxy or alkylthio (preferably methyl, ethyl, methoxy or methylthio), and R^{36} together with R^{37} represents 1,6-hexylene, ethylenethioethylene or a moiety of the general formula XIa



wherein X^3 and X^4 are the same or different and each represents sulphur or selenine, and R^{38} and R^{39} are the same or different and each represents alkyl, alkoxy or alkylthio (preferably methyl, ethyl, methoxy or methylthio), dioxapyrenes, dithiapyrenes, xanthylidenes and diacyanoimid-
 10 quinones and derivatives thereof, penanthrenequinone, tetrathiapentalene-2,5-dione, N,N,N,N-tetramethyl-1,4-phenylenediamine, 2,3,5,6-tetramethyl-1,4-phenylenediamine, thianaphthene, thianthene $\Delta^{4,4}$ -bithiopyran and 1,6-di-
 15 methyl-2,3,4,7,8,9-hexahydropyrido[2,3-g]quinoline have a potential utility as mediators.

Examples of specific compounds of formula VII are 2-methyl-1,4-benzoquinone, 5-chloro-2,3-dimethyl-1,4-benzoquinone, 5-hydroxy-2-ethoxy-3-methyl-1,4-benzoquinone, 2-
 20 (p-chlorophenylamino)-1,4-benzoquinone, 2-phenyl-1,4-benzoquinone, 2,5-diphenyl-1,4-benzoquinone, 3,6-dichloro-2,5-bis-(p-ethylphenylamino)-1,4-benzoquinone, 2-(o-nitrophenylthio)-1,4-benzoquinone, 2,3-dimethyl-1,4-napthoquinone, 6-chloro-2-hydroxy-7-methyl-1,4-napthoquinone, 6-
 25 methoxycarbonyl-1,4-napthoquinone, 2-carboxyethylthio-3-methyl-1,4-napthoquinone, 2,3-dimethyl-9,10-anthraquinone and 2,6-dimethoxy-9,10-anthraquinone. Further examples of specific compounds of formula VII are 2-methyl-1,4-napthoquinone, anthraquinone, 2,3-dichloro-1,4-napthoquinone 1,4-
 30 napthoquinone, 2-hydroxy-1,4-napthoquinone, 2-(hydroxymethyl)-9,10-antraquinone, phenylbenzoquinone, tert.butyl-1,4-benzoquinone, 1,5-dichloro-9,10-antraquinone, thymoquinone, 5,7,12,14-pentacenetetrona, vitamin K_1 , nuclear fast red, 2,5-diphenyl-1,4-benzoquinone, p-tolyl-1,4-benzo-
 35 quinone, 2,5-bis-(4-methylanilino)-3,6-dichloro-1,4-benzo-

quinone and (cyclohexylpropyl)-3,6-dimethyl-5-hydroxybenzo-quinone. Compounds of formula VII are either commercially available or can be produced by methods analogous to known methods (see Morrison and Boyd in "Organic Chemistry",
5 third Edition 1973, publ. Allyn and Bacon, Inc.).

Suitable salts of compounds of formula VIII are such with hydrophobic compounds, for example pyridinium TCNQ (hereinafter TCNQ designates 7,7,8,8-tetracyanoquino-
dimethane), triethyl ammonium TCNQ and propylpyridinium
10 TCNQ. Other suitable salts are $\text{TTF}^+\text{TCNQ}^-$, $\text{TMPD}^+\text{TCNQ}^-$ and Fc^+TCNQ^- . Herein, TTF designates tetrathiafulvalene, TMPD designates N,N,N,N-tetramethylphenylene diamine and Fc designates ferrocene. Compounds of formula VIII and salts
15 can be prepared as described by Besspalov et al., Russian, Chemical Reviews, 44 (1975), 1091 - 1108, Yamaguchi et al., J.Chem.Soc.Chem.Comm. (1982), 1065 - 1066, and Roberts et al., J.Org.Chem. 52 (1987), 2297 - 2299.

Examples of other specific mediators are 2,6-dimethyldioxapyran, 2,6-dimethyldithiapyran, dithiapyran,
20 dioxapyran and 2,6-dimethyl-4,8-diethyldioxapyran.

In compounds of formula IX, preferably 6, 7 or 8 of the symbols R^{19} , R^{20} , R^{21} , R^{22} , R^{23} , R^{24} , R^{25} , R^{26} and R^{27} are hydrogen. Examples of specific, preferred compounds
25 of formula IX are 10-methylphenothiazine, 4-nitro-3,7-bis-(dimethylamino)phenothiazine (methylene green), 3-dimethylaminophenothiazine, 3-dimethylamino-10-methylphenothiazine, 3-(morpholine-4-yl)phenothiazine and 2-chloro-10-dimethylaminopropylphenothiazine.

Preferred compounds of formula XI are tetramethyl-
30 dithiadiselenefulvalene, 2-(thiopyran-4'-ylidene)-1,3-dithiole and 2-cycloheptylidene-1,3-dithiole.

The electrode device comprises a sensor electrode, a reference electrode and a counter electrode. In a preferred embodiment two sensor electrodes are present. The electrode
35 comprises an electrically conductive material, together

with glucose oxidase and the mediator. The electrically conductive material is a carbon-based material such as surface-oxidized graphite particles of a size of up to 50 μm , preferably 1 - 20 μm .

5 According to one aspect of this invention, the mediator has a low water solubility. If the mediator is in the reduced form in the paste, the mediator preferably has a solubility in water in the range from about 1 μM to about 10 mM. If the mediator is in the oxidized form in the
10 paste, the mediator preferably has a solubility in water in the range from about 1 μM to about 100 mM.

 The solubility of a certain mediator in the pasting material may be high, low or medium. Herein the statement that the mediator has a high solubility in the pasting
15 material designates that the mediator is dissolved in the pasting material. Within the context of this invention, a high solubility of the mediator is a solubility of more than about 80 mM in the pasting material.

 In a preferred embodiment of this invention, the
20 mediator is wholly or partially present in crystalline form in the pasting material. This is the case when the mediator has a low or medium solubility in the pasting material. Within the context of this invention, a low solubility of the mediator is a solubility of less than about 1
25 mM in the pasting material. Surprisingly, the use of mediators in crystalline form give superior results. Preferably, homogeneous crystals are used.

 In a preferred embodiment, the electrically conductive material is a carbon-based paste containing a pasting
30 ing material. Useful pasting materials are non-polar substances which are substantially immiscible with water, such as paraffin, paraffin oil, silicone oil etc., paraffin oil being preferred.

 The pasting material constitutes a support and a
35 dispersion medium for the mediator and regulates the release of the mediator in a controlled way during the measurement. Furthermore, the pasting material serves as a

cohesive medium by filling the interstices between the graphite particles.

The enzyme preferred in the method according to the invention is glucose oxidase, which is an oxidoreductase enzyme of the classification EC 1.1.3.4. However, other enzymes which are oxidoreductases may be used, for example theophyllin oxidase, alcohol oxidase, lactate oxidase, cholesterol oxidase, glucose dehydrogenase, L-amino acid oxidase or a glycolate oxidase.

The glucose oxidase will be attached to the surface-oxidized graphite particles and/or immobilized on the latter particles by means of a treatment involving the use of a carbodiimide reagent such as, for example, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (= N-cyclohexyl-N'- β -[(N-methylmorpholino)ethyl]-carbodiimide p-toluenesulfonate salt). The glucose oxidase is present in the sensor electrode in an amount of at least 500 IU per gram of graphite, preferably in an amount of at least about 1000 IU, such as at least about 2000 IU, more preferably at least about 4000 IU, most preferably at least about 6000 IU per gram of graphite, and in particular about 8000 IU per gram graphite.

An essential feature of the sensor electrode is that the glucose oxidase and the mediator are uniformly or homogeneously distributed in the carbon-containing paste. In this way it is possible to cut off a thin, cross-sectional slice of the electrode and at the same time ensure that the correct and sufficient amount of glucose oxidase and mediator is present at the working surface of the electrode, the working surface of the sensor electrode being the surface which is exposed to the glucose-containing aqueous medium, such as a blood sample.

In a preferred embodiment of the sensor electrode for determination of glucose, two sensor electrodes are present in the electrode device. The two sensor electrodes can be similar or different. When the sensor electrodes are different, one electrode comprises the glucose oxidase in

an active form and the other comprises the glucose oxidase in an inactive form which has been inactivated by heat denaturation at 50°C for 24 hours.

The advantage of using two similar sensor electrodes for the measurements is that it is possible in this way to obtain two responses, one from each sensor electrode. The responses should be substantially equal if the measurement is performed correctly. Different responses from two similar electrodes is an indication of an unreliable measurement due, for example, to inadequate contact between the sample and the electrodes.

The advantage of using two different sensor electrodes, one containing the active enzyme and the other one containing the inactivated enzyme, is that it is possible in this way to obtain a blank response from the electrode containing the inactivated enzyme, whereby response from interfering substances can be subtracted from the measurement performed at the sensor electrode containing the active glucose oxidase.

In the method according to the invention, the measurement of the concentration of glucose in a sample of an aqueous medium is performed at a potential between the sensor electrode and a reference electrode in communication with the sample of the aqueous medium which is in a range which is about 0 - 250 mV when the reference electrode is an Ag/AgCl reference electrode working at a concentration of chloride ions of about 145 meq/l, or, when the reference electrode is different therefrom, which is in a range corresponding to a range of about 0 - 250 mV measured versus an Ag/AgCl reference electrode working at a concentration of chloride ions of about 145 meq/l.

A sensor electrode for the determination of glucose may be prepared in the following manner:

The carbon particles, preferably graphite particles, are surface oxidized by heating, for example, at 100°C for a predetermined period in a well ventilated, continuously rotated vessel in the presence of dry atmospheric

air. The surface-oxidized graphite particles are activated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate. The activation is performed by mixing the graphite particles with the 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate dissolved in an aqueous buffer solution, for example, an acetate buffer of pH value 4.76, followed by continuous stirring for about 2 - 4 hours at room temperature. After stirring, the mixture is washed several times (for example 4 - 7 times) with distilled water until an approximately neutral pH value (for example 5 - 7), and the carbodiimide-activated graphite particles are dried by evaporation of the water, for example by forced evaporation using a fan, or under reduced pressure, at room temperature or by lyophilization. Before immobilization of the glucose oxidase on the carbodiimide-activated graphite, the glucose oxidase is dissolved in an aqueous buffer having a suitable pH value at which the enzyme is sufficiently stable, and containing a coupling reagent for coupling the enzyme to the carbodiimide-activated graphite, for example a phosphate buffer having a pH value of 7.3 and containing 4 % (weight/weight) glutaraldehyde (i.e. glutaric dialdehyde). The immobilization is performed by suspending the carbodiimide-activated graphite particles in the buffer containing the enzyme and the coupling reagent. The suspension is stirred continuously for about 16 hours at 4°C followed by removal of the water by evaporation, for example at reduced pressure, at room temperature or by lyophilization. Using glutaraldehyde as coupling reagent the enzyme, preferably glucose oxidase, becomes covalently bonded to the graphite particles. The enzyme may, however, also be physically adsorbed to the surface of the particles.

In the following the term "GOD-containing graphite" is used to mean carbodiimide-activated graphite particles comprising the glucose oxidase, the particles being prepared in the manner described above.

The GOD-containing graphite is sieved through a 48

mesh (mesh size corresponding to a sieve having an opening of 297 μm) and the sieved material is then mixed with a sufficient amount of the non-polar pasting material, i.e. paraffin oil, in which the mediator has been dissolved. The amount of the mediator in the paraffin oil is larger than the required and final amount in the sensor electrode. The reason for this is that it should be possible to obtain a dispersion of GOD-containing graphite in the mediator solution having a consistency which allows the appropriate channel(s) in the electrode device body to be filled. The excess amount of the mediator solution is easily removed after filling of the channel(s) of the sensor electrode in the electrode device body by centrifugation.

To fill the sensor electrode channels(s), the electrode device body is placed in a special holder having a small funnel-shaped reservoir at the top. The dispersion containing the GOD-containing graphite and the mediator in paraffin oil solution is then poured into the reservoir. The electrode device body equipped with the filled reservoir is then centrifuged at about 15,000 - 50,000 $\times g$ for about 5 - 30 minutes, the electrode body device being positioned in an inverted position during centrifugation (i.e. that end of the device from which slices later are removed being uppermost, the funnel-shaped reservoir being inserted in that end). By the centrifugation the air is displaced from the channels and the GOD-containing graphite particles sediment, the interstices between the particles thus being filled with the mediator in paraffin oil, and the excess amount of the mediator paraffin oil remaining as a supernatant in the upper part. After centrifugation the excess of the mediator is easily removed and the surface of the sensor electrode is levelled with the surface of the electrode device body by cutting off a suitable cross-sectional portion of the electrode device.

The advantages achieved by using the above-mentioned method of preparing and filling the sensor electrode channel(s) in the electrode device body is that the compo-

nents of the sensor electrode(s) become packed uniformly or homogeneously in the graphite-containing paste, the interstitial spaces between the GOD-containing particles being filled with the mediator in paraffin oil such that substantially no air bubbles remain within the sensor electrode(s).

Stability test

The stability of a paste with a certain mediator can be determined using the following method:

10 The mediator is dissolved or mixed with paraffin and, thereafter, the enzyme coupled to graphite is added. The mixture is homogenized giving a paste. This paste is filled into electrode holders of plast giving so-called sticks. These sticks are tested using 0 and 10 mM glucose
15 standards in order to characterize the activity of these sticks in relation to glucose. Thereafter, the paste containing sticks are stored at a constant temperature, for example, 4, 20, 35 and 54°C. After a certain period of time, for example 1 month, the sticks are tested using the
20 0 and 10 mM glucose standards and the activities measured are compared with the activity measured at originally. Those mediators present in the sticks for which the change of measurements for glucose are the lowest, are considered to be the mediators most stable in the system (pasta) in
25 question.

Standard methods for evaluation of mediators

1) A paste containing GOD-immobilized graphite, paraffin and mediator is prepared. If the mediator has a big solubility in paraffin then 10 mg of mediator is used per ml of
30 paraffin. If the mediator has a little solubility in paraffin then 50 mg of crystals are added per ml of paraffin in order to obtain a homogeneous mixture before graphite is added. The paste obtained is filled into sticks.

- 2) The activity of the paste is determined in a cyclic voltametri equipment where the current is measured as a function of the potential (CV sweep). Five concentrations of glucose are measured, i.e., 0, 5, 10, 15 and 20 mM.
- 5 Based upon the results obtained, the following is evaluated:
- a) whether the mediator can be oxidized and reduced by the graphite electrode,
 - b) whether the mediator can be reduced of GOD,
 - 10 c) the optimal potential for the electorde to be used,
 - d) whether a lineary response as a function of the concentration of glucose can be obtained using the concentration of mediator in question,
 - e) whether the concentration of the mediator can be opti-
 - 15 mized.
- 3) The response as a function of time at the optimal potential is to be measured. The time interval within which the response represents a stable response for the concentration of glucose is to be evaluated.
- 20 4) The stability of the mediator in the paste question is determined.

The concentration of the mediator in the sample phase can be determined using the method described in Bioelectrochemistry and Bioenergetics 8 (1981), 103 - 113.

25 Reference electrode

The reference electrode can be any physically suitable reference electrode of a conventional type, preferably a silver/silver chloride reference electrode or a variant thereof comprising an aqueous solution of potassium chloride (for example a 1 M solution) in a gel (for example an agarose gel) and at the bottom having a silver/silver chloride electrode. A silver/silver chloride electrode is preferred, the electrode comprising a silver wire on which

a layer of silver chloride has been established by immersion in ferric chloride, or by an electrolytic treatment in 0.1 M hydrochloric acid. The silver wire is rather thin and has preferably a diameter of about 100 - 300 μm , in particular about 200 μm . Alternatively, the reference electrode can comprise an appropriate number of silver chloride coated silver wires, each wire having a diameter of about 20 - 100 μm , preferably about 30 μm .

Following removal of a cross-sectional slice of the electrode device prior to performing a measurement it is clear that the freshly exposed cross-sectional surface of the silver wire is no longer covered by silver chloride. However, upon exposure to a sample of an aqueous medium which contains at least a moderate concentration of chloride ions (such as whole blood or urine) the latter cross-sectional surface rapidly becomes coated with an adequate layer of silver chloride by reaction between the silver from the silver wire and the chloride present in the aqueous sample. The chloride present in the aqueous sample may arise from dissolution of silver chloride from the periphery of the exposed silver surface of the reference electrode itself and/or from the content of chloride ion in the sample. In the case where the aqueous sample itself contains only a small concentration of chloride ions it is thus advantageous to use a reference electrode comprising a number of silver wires, whereby a greater surface area of the silver chloride from the periphery of the reference electrode surface is available to release chloride ions to the aqueous sample, resulting in the formation of the necessary layer of silver chloride on the cross-sectional surface of the silver wire.

Alternatively, the reference electrode may be made of any sliceable plastic material in which is embedded a powder comprising small silver and silver chloride particles.

The reference electrode is suitably prepared by inserting the silver wire(s) on which the silver chloride

layer has been established into the reference electrode channel of the electrode device body. The silver/silver chloride electrode is then fixed into position in the channel by injecting, for example, two-component epoxy adhesive into the free space in the channel.

Counter electrode

The counter electrode is preferably a silver wire of diameter about 100 - 300 μm , preferably about 200 μm . It may also be made of any suitably electrically conductive sliceable polymer-based material, for example a plastic material, or an electrically conductive carbon-based paste, for example a graphite-based paste.

During the measurement, the counter electrode delivers an electrical current which counterbalances the current generated at the sensor electrode as a result of the electrochemical reaction at the surface exposed to the sample, and as a result a fixed, constant potential between the sensor electrode and the reference electrode can be maintained.

Operation of the sensor electrode of an electrode device according to the invention at relatively low and constant potential is very important if interference from oxidizable substances other than glucose present in the blood is to be avoided.

In addition, the measurement must be reproducible and accuracy should be high, particularly at low glucose concentrations (as would be the case for a patient in hypoglycemic coma). The patient himself should be able to check the correct functioning of the electrode device by using a single test solution and finally it should be possible to manufacture the sensor on a large scale to strict specifications.

Any novel feature or combination of features described herein is considered essential to this invention.

This invention is further illustrated by the following examples which, however, are not to be construed as

limiting.

Example 1

Glucose oxidase (GOD) (E.C.1.1.3.4.) was immobilized on graphite particles by the following procedure: 10 g of graphite was oxidized in air at 100°C for 24 hours. The surface oxidized graphite particles were activated with 500 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate in 100 ml of 0.1 M acetate buffer (pH 4.76) for 2 hours, then washed with distilled water until an approximately neutral pH value and dried. The activated graphite was mixed with 30 ml of 0.01 phosphate buffer (pH 7.3) containing 80,000 IU GOD and 4% glutaraldehyde for 16 hours at 4°C, dried and sieved through a 48 mesh to obtain uniform particles.

1 g of paraffin oil containing 5% Methylene green (4-nitro-3,7-bis(dimethylamino)phenothiazine) was mixed carefully with 2 g of GOD modified graphite to a uniform pasta.

The pasta was filled into a plastic rod in which it was possible to cut a thin cross-sectional slice to introduce a new well-defined electrode area. Before each measurement, the electrode surface was renewed. The working electrode area used was 3 mm².

In the study of the working electrode, an Ag auxiliary electrode and an Ag/AgCl reference electrode was used. In a cyclic voltammogram, a catalytic current from the reoxidation of the mediator was observed beginning at -50 mV and being stable between 50 and 400 mV. The current was proportional to the concentration of glucose used.

Chronoamperometry was performed at 100 mV using the integrated current from 30 to 60 seconds after applying the potential. A linearity up to 40 mM glucose was obtained. The current was 500 nA/(sec*mM). The signal was not affected by the oxygen concentration in the solution. Reference pasta without Methylene green was not affected by

different glucose concentrations.

Example 2

Comparison between selected mediators.

Data in the table are obtained using the same procedure as in Example 1.

The following abbreviations are used: Methylene green = MG, Mendola blue = MB, 1-dimethylamino-4-morpholinobenzene = MDMP, 1,4-dimorpholinobenzene = BMP, 1,4-dipiperidinobenzene = BPP, 1-morpholino-4-piperidinobenzene = MPP.

	MG	MB	MDMP	BMP	BPP	MPP
Concentration in pasta (%)	5	5	2	2	0.5	2
15 Redoxpotential at GCElectrode ¹	-149	-175	124	246	141	166
Catalytic interval beginning at (mV) ¹	-50	-50	-20	50	0	20
20 Working potential ¹	100	100	100	200	150	100
Signal to noise ration ²	22.5	4.8	8.3	5.6	4.7	10
Linearity in mM	40	40	20	20	20	20
25 SD% of 100 measurement ²	7.5	6.5	4.5	6.5	3.5	4.0

¹Ag/AgCl ref., ² at 7mM glucose.

Example 3

Stability test of selected mediators.

Data are obtained using the same basis procedure as in Example 1. Rods with selected mediators were stored at different temperature to accelerate processes which could give new analytical performance.

The same abbreviations as in Example 2 are used.

Mediator:		MG		MDMP		BMP		BPP		MP		
<hr/>												
10	Time:	0	75	0	72	0	72	0	78	0	70	
<hr/>												
15	Temp.											
	4°C	a	13.7	13.2	17.2	16.9	20.2	21.2	12.3	12.4	11.7	8.8
20		b	10.0	14.3	16.2	18.1	21.3	19.3	11.1	13.7	9.2	10.5
	20°C	a	14.9	14.3	16.4	17.4	18.5	21.4	10.0	11.0	10.0	11.9
25		b	12.5	15.4	16.0	15.4	20.5	23.3	10.5	11.1	7.2	9.3
	35°C	a	12.1	11.2	15.4	15.7	20.0	18.0	9.1	8.7	7.0	9.0
25		b	13.3	12.2	13.4	14.5	16.7	20.3	10.6	10.4	9.0	9.5
	54°C	a	15.8	10.4	17.4	6.9	18.4	15.0	11.1	6.7	12.5	4.1
25		b	12.8	12.6	14.4	8.5	20.5	14.6	10.3	5.6	11.3	4.7

Taking the reproducibility from slice to slice and from rod to rod into account, it can be concluded that all

pasta are stable within at least 70 days at 35°C. From the data at 54°C, it can be extrapolated that the stability at 4°C will be at least 2 years.

Example 4

5 Mediators for a theophylline assay.

Theophylline oxidase was obtained from GDS Diagnostics, Division of GDS Technology, Inc., 25235 Leer Drive, P.O. Box 773 Elkhart, IN 46515, USA. The GDS Enzymatic Theophylline Reagent kit was used. The reagent B which
10 contained the enzyme Theophylline oxidase was dialyzed against a phosphate buffer at pH 7.0 and the enzyme was lyophilized and coupled to graphite as in Example 1. The mediators used in Example 2 and dimethylferrocene (DMF) and tetrathiafulvalene (TTF) were tested in the system. DMF and
15 TTF were used in 0.1 M and 0.06 M in paraffin. DMF was measured with a potential at 110 mV vs. Ag/AgCl reference electrode and for TTF 60 mV was used. For the rest of the mediators the potentials as in Example 2 were used. The current density obtained differs from the glucose reaction
20 in Example 1. Typically, the current density was in the range 1 - 10 $\mu\text{A}/\text{cm}^2/\text{mmol}$. An oxygen dependence was observed at low concentrations.

Example 5

Mediators for lactate and alcohol assays.

25 Lactate oxidase and alcohol oxidase were obtained from Sigma and the enzymes were used as in Example 1. The same mediators as in Example 4 were tested in the electrodes. The best response for the lactate system was obtained with Methylene green, which gave a current density
30 at 4 $\mu\text{A}/\text{cm}^2/\text{mM}$ at 100 mV vs. Ag/AgCl reference electrode. For the alcohol system ethanol was used as enzyme substrate. The current density was much lower than for the

lactate system and none of the tested mediators worked optimally. Other mediators successfully tested with glucose oxidase are expected to give higher current densities due to differences in hydrophobicity and hydrophilicity.

CLAIMS

1. A biochemical process involving electron transfer between a redox system and an electrode comprising a paste of electrically conductive particles and a pasting material
5 and a charge transfer mediator, wherein the mediator is a compound of the general formula X mentioned above with the definition of the symbols stated above.
2. Process, according to Claim 1, wherein the mediator is 1,4-diamino-2,3,5,6-tetramethylbenzene, N,N,N-tetra-
10 methyl-1,4-phenylenediamine, 1,4-bis(dimethylamino)benzene, 1-dimethylamino-4-morpholinobenzene, 1,4-dimorpholinobenzene, 1-morpholino-4-piperidinobenzene, 2,5-diethyl-1,4-bis-(dimethylamino)benzene, 1,4-dipiperidinobenzene, N-(4-morpholinophenyl)hexahydroazepine, 4-nitro-3,7-bis(dimeth-
15 ylamino)phenothiazine (= methylene green) and Meldola's Blue (= 7-dimethylamino-1,2-benzophenoxazinium salts, for example chloride or sulphate).
3. Process, according to Claim 1 or 2, wherein the mediator wholly or partially is present in crystalline
20 form in the pasting material.
4. Use of benzene derivatives with the general formula X mentioned above with the definitions of the symbols stated above as a charge transfer mediator, preferably those stated in Claim 2.
- 25 5. Any novel feature or combination of features described herein.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00308

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: G 01 N 33/48, G 01 N 27/327		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	G 01 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²	Relevant to Claim No.¹³
Y	WO, A1, 8910395 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 2 November 1989, see claim 12 --	1-5
Y	WO, A1, 9010861 (NOVO-NORDISK A/S) 20 September 1990, see page 1, line 2 - line 10; page 1, line 57; claims 1,10,11 --	1-5
Y	Chemical Abstracts, volume 92, no. 15, 14 April 1980, (Columbus, Ohio, US), Baccarini-Melandri, Assunta et al: "The stimulation of photophosphorylation and ATPase by artificial redox mediators in chromatophores of Rhodopseudomonas capsulata at different redox potentials ", see page 378, abstract 125207d, & J.Bioenerg.Biomembr. 1979, 11(1-2), 1- 16 --	1-5
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
14th January 1992		1992 -01- 16
International Searching Authority		Signature of Authorized Officer
SWEDISH PATENT OFFICE		<i>Carolina Palmcrantz</i> carolina Palmcrantz

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Analytical Chemistry of the USSR. Part 1, Vol. 45, No. 9, 1990 J. Labuda: "Chemically modified electrodes as sensors in chemical analysis", see page 445 - page 455 especially table 2 page 451 ---	1-5
A	EP, A2, 0125137 (GENETICS INTERNATIONAL, INC.) 14 November 1984, see the whole document -- -----	1-5

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers.....⁵....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The wording of claim 5 is not clear and concise.
See article 6.

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00308**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 30/11/91
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO-A1- 8910395	89-11-02	EP-A-	0409896	91-01-30
		FR-A-	2630546	89-10-27
		JP-T-	3503931	91-08-29

WO-A1- 9010861	90-09-20	AU-D-	5268290	90-10-09
		CA-A-	2028840	90-09-10

EP-A2- 0125137	84-11-14	AU-B-	564494	87-08-13
		AU-B-	564495	87-08-13
		AU-B-	569076	88-01-21
		AU-B-	580257	89-01-12
		AU-D-	2775184	85-01-31
		AU-D-	2775284	85-01-31
		AU-D-	2775384	84-11-08
		AU-D-	2775484	84-11-08
		CA-A-	1218704	87-03-03
		CA-A-	1219040	87-03-10
		CA-A-	1220818	87-04-21
		CA-A-	1223638	87-06-30
		EP-A-B-	0125136	84-11-14
		EP-A-	0125139	84-11-14
		EP-A-	0125867	84-11-21
		JP-A-	60017345	85-01-29
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		JP-A-	60017347	85-01-29
		JP-A-	60017360	85-01-29
		US-A-	4711245	87-12-08
		US-A-	4758323	88-07-19
		AU-B-	572138	88-05-05
		CA-A-	1226036	87-08-25
		EP-A-	0127958	84-12-12
		EP-A-	0351891	90-01-24
		EP-A-	0351892	90-01-24